High-Molecular Intestinal Alkaline Phosphatase in Chronic Liver Diseases

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The presence of high-molecular intestinal alkaline phosphatase (HIALP) different from bone ALP detected in the $\alpha_2\beta$ region was recently clarified. In this study we used a novel method in which HIALP was detected after conversion to ALP₅ by protease to investigate the clinical significance of the appearance of HIALP in patients with chronic liver disease. The subjects were 241 patients with chronic liver disease. When a decrease in ALP₃ in the $\alpha_2\beta$ region and an increase in ALP₅ in the β region were noted, the patient was judged HIALP-positive. In the patients with chronic liver disease, the total ALP activity (T-ALP) increased with progression of the pathology in the order of chronic hepatitis (CH), liver cirrhosis (LC), and hepatocellular

carcinoma (HCC). HIALP appeared in 22.4% and 49.3% of patients with CH and LC, respectively, but the positivity rate decreased to 30.4% in HCC. As autoimmune liver diseases, primary biliary cirrhosis (PBC) and autoimmune hepatitis (AIH) were investigated. T-ALP was lower in PBC + AIH than in LC and HCC, but the HIALP-positive rate was high (44.4%). The HIALP-positive rate was dependent on ABO blood groups, and was high in blood groups B and O. In conclusion, the HIALP-positive rate was particularly high in patients with chronic liver disease, and was related to the pathological progression, which suggests that the method is clinically useful. *J. Clin. Lab. Anal.* 21:133–139, 2007.

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INTRODUCTION

Analysis of alkaline phosphatase (ALP) isoenzymes using agarose gel or cellulose acetate membranes has been widely used as a routine examination because of its simplicity and the capability of handling many samples. However, a band sharper than bone ALP frequently overlaps bone ALP in the $\alpha_2\beta$ region in many cases of liver cirrhosis (LC). Since this band is clinically unlikely to be the bone type, methods to separate and identify the organ of origin are needed.

It was recently reported that this band is HIALP. In 1988 Van Hoof et al. (1) employed neuraminidase treatment and polyclonal and monoclonal antibodies against the intestinal type and confirmed that the band overlapping bone ALP on agarose electrophoresis is an intestinal variant of ALP. In 1998 Hoshino et al. (2) confirmed that this band is a high-molecular band by polyacrylamide gel electrophoresis (PAGE), and

Matsushita et al. (3) reported that the band could be separated from bone ALP by conversion to ALP₅ by subtilisin treatment.

We confirmed the usefulness of a new method in which HIALP was converted to ALP₅ by protease treatment and electrophoresed on agarose gel. In this study we measured HIALP in sera from patients with

Abbreviations: ALP, alkaline phosphatase; IALP, intestinal alkaline phosphatase; HIALP, high molecular intestinal alkaline phosphatase; ALP₅, normal size intestinal alkaline phosphatase; NLD, non-liver disease; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; PBC, primary biliary cirrhosis; T-ALP, total alkaline phosphatase activity.

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chronic liver disease using this method. This method is capable of detecting and quantifying HIALP by electrophoresis of sera before and after protease treatment in adjacent lanes. Using this method, we compared the appearance of HIALP with T-ALP and the appearance of ALP₅ with regard to their relationship with the pathology.

MATERIALS AND METHODS

Subjects

The subjects of this study were 241 inpatients and outpatients with chronic liver disease who were being treated at Mie University Hospital. Eighty-five patients had chronic hepatitis (CH), 69 had liver cirrhosis (LC), 69 had hepatocellular carcinoma (HCC), and 18 had PBC and AIH. Ten healthy subjects were also included in the study. The autoimmune liver diseases were autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC). Since there were only seven and 11 patients with AIH and PBC, respectively, and the HIALP-positive rate was similar in these two groups, these patients were combined in the investigation.

Methods

T-ALP was measured with the use of the Vitros 250 Chemistry System and Test Methodology ALKP (Ortho-Clinical Diagnosis, Raritan, NJ, USA). The reference normal value was 100–350 U/L.

For analysis of ALP isoenzymes, we investigated the method using agarose gel, Quick Gel ALP, and an ALP reagent (Quick ALP) that was developed for an electrophoresis processing analyzer (Epalyzer 2; all from K.K. Helena Kenkyujo, Saitama, Japan). Regarding the measurement principle, formazan pigments produced using 2-indoxyl-phosphate disodium salt as the substrate and Nitroterazolum blue as the color developer were fractionated by densitometry at a wavelength of 570 nm.

For detection of HIALP, the samples were pretreated with protease (*Streptomyces griseus*, from K.K. Helena Kenkyujo). Serum and protease were mixed at a ratio of 5:1, kept at room temperature for 30 min or longer, and electrophoresed with untreated serum in adjacent lanes. HIALP was converted to ALP₅ by protease. When a decrease in ALP₃ and an increase in ALP₅ were noted, the patient was judged HIALP-positive. The measurement conditions were as follows: protease treatment for 30 min, electrophoresis at 250 V for about 20 min, staining for 30 min, discoloring fixation for 13 min, and washing with water for 30 min.

Student's *t*-test was used to assess the significance of differences.

RESULTS

Detection of HIALP by Agarose Gel Electrophoresis

Serum and protease samples from the subjects were mixed at a ratio of 5:1, kept at room temperature for 30 min or longer, and electrophoresed with untreated serum in adjacent lanes. When a decrease in the sharp band overlapping ALP₃ and an increase in ALP₅ were visually detected in the zymogram, the sample was judged HIALP-positive. Fig. 1 shows the patterns of ALP isoenzymes in fractionation of HIALP by densitometry at 570-nm wavelength. The upper and lower columns represent sera before and after protease treatment, respectively. The anode is on the left side, and ALP₁, ALP₂, ALP₃, and ALP₅ were separated in this order from the anode side. ALP₁ was the high-molecular liver type, and ALP₂ was the liver type. ALP₃ was the high-molecular intestinal type overlapping the bone type in the upper column, and the bone-type alone in the lower column. ALP₅ is the normal-size intestinal type.

Case 1 was a 52-year-old male CH patient with 457 U/L T-ALP and blood group B. Case 2 was a 78-year-old male LC patient with 482 U/L T-ALP and blood group O. Case 3 was a 70-year-old male LC patient with 445 U/L T-ALP and blood group O.

There were almost no changes in ALP₁ and ALP₂, but ALP₃ was decreased and ALP₅ was increased. In case 1, the ALP₃ decreased from 55.3% to 46.0%, and ALP₅ increased from 27.8% to 38.4%. In case 2, ALP₃ decreased from 30.0% to 23.9%, and ALP₅ increased from 34.3% to 43.6%. In case 3, ALP₃ decreased from 40.8% to 24.8%, and ALP₅ increased from 45.2% to 59.7%. We confirmed that the %CV is under 5% (data not shown).

Comparison of T-ALP and HIALP-Positive Rate Among Diseases

T-ALP is compared among the diseases on the upper of Fig. 2. The level increased with progression of the pathology from NLD, CH to LC and HCC. There were significant differences between NLD, CH and LC, HCC, and PBC+AIH.

The HIALP-positive rate is compared among the diseases in the lower part of Fig. 2. The positivity rate was 22.4% in CH and was the highest (49.3%) in LC, but decreased to 30.4% in HCC. The positivity rate in PBC+AIH was 44.4%.

Relationship Between the Appearance of Two Types of HIALP and ALP₅

In the comparison of the appearance of HIALP and ALP₅, ALP₅ was not detected in one CH patient out of

Fig. 1. Changes in ALP isoenzyme patterns in three patients with HIALP before and after protease treatment. Upper: before protease treatment; lower: after protease treatment.

the HIALP-positive patients, but ALP₅ was detected in 17 CH, 34 LC, and 21 HCC patients. In contrast, HIALP was detected in 26.1–35.3% of normal-size ALP₅-positive patients.

The relationship between the appearance of HIALP and ALP₅ was investigated in 85 CH, 69 LC, and 69 HCC patients. The patients were classified based on the presence or absence of HIALP, and HIALP-negative patients were classified based on the presence or absence of ALP₅. In the HIALP-positive patients, ALP₅ was absent only in one patient, and this patient was excluded from the classification. Thus the HIALP-positive patients were also ALP₅-positive. In CH, T-ALP was significantly increased in ALP₅-positive and HIALP-positive patients. In LC, T-ALP was slightly increased in ALP₅-positive patients and increased in HIALP-positive patients, but the increases were not significant. In HCC, T-ALP was slightly increased in ALP₅-positive and HIALP-positive patients (Fig. 3).

Relationship Between the Appearance of HIALP and ABO Blood Groups

The relationship between the appearance of HIALP and ABO blood groups was investigated in ABO blood-typed patients (Fig. 4). Almost no HIALP was detected in patients typed A and AB. HIALP was positive in one type-A patient in CH (4.3%), three type-A patients and

one type-AB patient in LC (16.7%), and one type-A patient in HCC (3.1%). The HIALP-positive rate was slightly higher in blood groups A and AB in LC. The positivity rate was high in blood groups B and O in all chronic liver diseases, but slightly low in CH (35.5%, 11/31). The positivity rate in blood groups B and O was the highest in LC (72.2%, 26/36), and slightly lower in HCC (58.1%, 18/31).

Although there were fewer PBC+AIH patients, the HIALP-positive rate was 86.7% (7/9), which shows that HIALP appeared at a higher rate than in LC, and all positive patients were blood type B or O.

Relationship Between T-ALP and ABO Blood Groups

The relationship between T-ALP and ABO blood groups was compared among the diseases in blood-typed patients (Fig. 5). T-ALP was higher in blood groups B and O than in A and AB in CH, LC, and HCC, and the difference was significant in LC.

DISCUSSION

IALP (ALP₅) migrates to the β region on agarose or cellulose acetate membrane electrophoresis, and it is known to have a high positivity rate in LC. High positivity rates of ALP₅ in LC (4) and renal failure (5)

Fig. 2. T-ALP and positive rate of HIALP in chronic liver diseases.

have been reported. A dependence of ALP₅ on ABO blood groups has also been reported (6,7). Langman et al. (6) reported that ALP₅ was detected in about 38% of blood groups B and O and healthy secretors, but not in blood groups A and AB and nonsecretors of all blood groups, showing its blood-group dependency. All of these reports were about ALP₅.

Several studies recently reported that HIALP migrates to the $\alpha_2\beta$ region on agarose or cellulose acetate membrane electrophoresis, and is dependent on ABO blood groups (1–3,9–12). Van Hoof et al. (1) and Kuwana and Rosalki (9) detected variant IALP after pretreatment with neuraminidase or antibody against

the intestinal type, using agarose gel and cellulose acetate membrane, respectively, for the support medium. Hoshino et al. (2) and Matsushita et al. (3) also analyzed the high molecule by PAGE. Matsushita et al. (3) reported that the band as an intestinal variant and HIALP were the same isoform. Based on the absence of a sugar chain due to the absence of change in migration after neuraminidase treatment, and its identification as an intestinal type by treatment with antibody (1,9), the band detected in the $\alpha_2\beta$ region and overlapping the ALP₃ was determined to be HIALP.

This band was dependent on ABO blood groups, as was ALP₅, and appeared at a high rate in blood groups

Fig. 3. Relationship between T-ALP and IALP.

Fig. 4. Relationship between HIALP and ABO blood groups.

B and O (secretor) (3,8–13). The positivity rate of this band was high in diseases with a high positivity rate of ALP₅, and the appearance in LC has been most frequently reported (8,12,14). Its appearance in diabetes (9) and renal failure (9,14) has also been reported.

There have been many reports of changes in IALP (ALP₅ and HIALP) in subjects who consumed a high-fat diet (6,15–17). IALP increased after meals and was associated with blood groups. The positivity rate increases in the order of blood groups B and O secretors,

Fig. 5. Relationship between total T-ALP and ABO blood groups.

AB secretors, and A secretors, but IALP does not appear in nonsecretors. The IALP that increased in subjects on a high-fat diet was ALP_5 , and almost no change was noted in HIALP.

The previously reported HIALP measurement methods are useful for research but are too complex for use as a routine test. In this study we employed a novel method that uses agarose gel for the support medium, in which HIALP is converted to ALP_5 by protease pretreatment, and investigated the positivity rate of HIALP in various chronic liver diseases (CH, LC, HCC, PBC, and AIH). The positivity rate was associated with ABO blood groups, as it is in healthy adults, but varied among the diseases.

The mechanism of increase in IALP may be a decreased catabolism in the liver, rather than inflow of IALP into the circulation. In LC, decreased liver function may decrease ALP_2 production and catabolism of IALP, changing the hemodynamics, and decrease ALP_2 and increase HIALP/ ALP_5 .

Protease specifically degrades HIALP but does not degrade ALP_1 , although it is a high molecule. ALP_1 is a complex of liver ALP and lipid. It is a giant molecule with a molecular weight of more than 30 kDa, and is derived from hepatocytes. In 1981 Sakuma et al. (18) named a band specifically detected in the $\alpha_2\beta$ region in LC patients, and concluded that the molecule was a high-molecular form of IALP (ALP_5) based on its

enzymatic properties. This band may be consistent with HIALP successively reported after 1988. When HIALP was subjected to gel filtration in the presence of a surfactant, as was ALP_1 , the molecular weight was decreased, which suggests that the molecule forms a complex with lipids. However, the molecular weights of some components were not decreased, and their molecular weights were estimated to be 300,000–400,000. Thus they are not giant molecules, which suggests the presence of forms other than complexes with lipids.

We consider that ALP_5 is bound to various substances, which increases the molecular weight, and forms HIALP, and the binding substances vary among diseases. HIALP appears at a high rate in chronic liver diseases, but it is accompanied by the appearance of ALP_5 in most patients (98.6%), suggesting polymerization of ALP_5 . The molecular weight of 2–3 polymerized molecules is almost consistent with HIALP.

ALP is considered to be normally present as a covalently bonded homodimer; however, a tetramer (two molecules) of liver ALP has also been reported (19). The presence of a hybrid heterotetramer consisting of intestinal and placental forms in cancer tissues and established cancer cells, in addition to a homotetramer, has also been reported (20). Based on the stereo structure, binding of two IALP molecules (tetramer) is possible.

An association of HIALP with pathological progression was noted. In a comparison of CH, LC, and HCC,

the HIALP-positive rate was relatively low (21.2%) in CH, and the rate was 20.0% when one ALP₅-negative patient was excluded. The overall ALP₅-positive rate regardless of appearance of HIALP was 53.3%. In LC, the HIALP-positive rate was 49.3% and the ALP₅-positive rate was the highest (75.4%). In HCC the HIALP- and ALP₅-positive rates were respectively 30.4% and 62.3% lower than those in LC. T-ALP increased with progression of the pathology, but the two types of IALP peaked in LC and slightly decreased in HCC. T-ALP increased due to the appearance of ALP₁ and an increase in ALP₂. In LC, ALP₅ was markedly increased, and ALP₂ was very low in some patients.

In chronic liver diseases, viral hepatitis B and C is clinically important. Hepatitis B is likely to become fulminant, but completely resolves in many cases. It progresses to LC in some patients within 3 years in most cases. In e-antigen-positive patients, HCC is likely to develop within a relatively short period. Many patients have hepatitis C, which progresses to CH, LC, and HCC after a long course. Most cases of HCC develop from LC; however, rare cases develop from CH, and some cases remain LC and do not develop HCC. For all cases, early discovery of progression from CH to LC and development of HCC from CH/LC is essential. At present, imaging diagnosis is generally more important tumor markers in order to diagnose HCC. However, imaging diagnosis is not capable of handling many cases because of the time of examination and performance of diagnosis, and its application is limited, considering the number of patients with hepatitis C and its long course.

Since IALP increases with progression from CH to LC and decreases in HCC, when the course is traced in patients (blood groups B and O in particular), the appearance or increase of IALP suggests progression from CH to LC, and an increase in T-ALP and a decrease in IALP suggest the development of HCC. Since HIALP is not affected by a high-fat diet or nutrition, it may be clinically useful. Since HIALP can be measured noninvasively, it can be frequently tested in many individuals, which is clinically significant. It is necessary to elucidate the pathology and mechanism of changes in IALP by tracing patients in blood groups B and O.

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